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Preclinical report

Dimethyladamantylmaleimide-induced *in vitro* and *in vivo* growth inhibition of human colon cancer Colo205 cells

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The effect of *N*-1-(3,5-dimethyladamantyl)maleimide (DMAMI) on the growth of Colo205 human colon cancer cells was examined both *in vitro* and *in vivo*. Flow cytometry analysis showed a decrease of G_2/M Colo205 cells at 4–6 h after treatment with DMAMI prior to accumulation of apoptotic cells at 24 h. Significant changes in cell morphology, i.e. shrinkage and chromatin condensation of cells, were observed after treatment with DMAMI. In the analysis of the apoptosis markers, it was found that the increase of Annexin V binding to membrane, peroxide radicals, dissipation of the mitochondrial membrane potential, and the activation of caspase-3, -8 and -9 were all evident at 4–6 h after treatment with DMAMI. *In vivo* analysis showed that treatment of Colo205 tumor-bearing SCID mice with DMAMI (230 mg/kg, intratumoral, once) resulted in rapid tumor damage that leads to significant tumor growth inhibition and no obvious acute toxicity. These results suggest that DMAMI has potential for local treatment of cancer. [© 2002 Lippincott Williams & Wilkins.]

Key words: Apoptosis, caspase, colon cancer, dimethyladamantylmaleimide, mitochondrial membrane potential, reactive oxygen species.

Introduction

Adamantane derivatives possess several attractive pharmacological activities such as antibacterial, antifungal, antiviral and anticancer effects; many investigators consider them as highly promising candidates in drug design.[1–4] *N*-1-adamantylmaleimide (AMI) has been synthesized, and shows anticancer activity in mice and inhibition of herpes simplex virus *in vitro*.[5] Our previous study has found that *N*-1-adamantylcitraconimide, AMI, *N*-1-(3,5-dimethyladamantyl)maleimide (DMAMI) and *N*-1-diamantylmaleimide exhibited growth-inhibitory activities *in vitro* against several cancer cell lines (Colo205, SC-M1, HepG2, SK-BR-3 and Molt-4),[6] and DMAMI exhibited specific activity against Colo205 colon tumor cells.[7] In addition, we have found that the *N*-substituents of maleimides with adamantyl substituents have a more potent antitumor activity than the maleimides with diamantyl substituents.[6,7] Recently, we have found that AMI exerts significant growth inhibitory activity *in vivo* against SC-M1 gastric cancer cells.[7] In determining the cellular targets of AMI in SC-M1 cells, we found that the conformational conversion of membrane protein of human gastric cancer SC-M1 cells from an α-helix to a β-sheet was not only time-dependent but also dose-dependent on the AMI concentrations used.[8] We also found that the expression of CD29 and CD54 on SC-M1 was decreased, and caspase-3 activity was increased during the early apoptosis induced by AMI.[9]

Colon cancer is the second leading cause of death from cancer in the US and most industrialized countries.[10] Although chemotherapy and radiation therapy have been attempted in either adjuvant or palliative treatments, adjuvant therapy is not effective in colon cancer patients. Nearly half of all patients with colon cancer still die of metastatic disease after curative surgery.[11] Therefore, developing new therapeutic drugs or techniques for colon cancer is a worthwhile task. Our previous results showed that

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DMAMI displayed highly selective cytotoxicity *in vitro* against Colo205 human colon cancer cells, with an IC_{50}=0.55 μg/ml, which is similar to adriamycin.[7] To the best of our knowledge, we report here the first example of DMAMI-induced growth inhibition *in vivo* against colon cancer cells. In this study, Colo205 cells were implanted s.c. in SCID mice to evaluate whether DMAMI can inhibit human tumor growth *in vivo*. In addition, we have characterized the biochemical and molecular events of DMAMI-induced cell death; these include morphologic change, cell growth inhibition, cell cycle progression, Annexin V binding, mitochondria membrane potential ($\Delta\Psi_m$) disturbance, reactive oxygen species production, and the activation of caspase-3, -8 and -9 in Colo205 colon cancer cells. The tumor tissue in SCID mice was damaged rapidly after treatment with DMAMI. These inhibition effects appear to be mediated by dissipation of the $\Delta\Psi_m$, and activation of the pro-caspase-3, -8 and -9. The elevation of Annexin V binding, peroxide radicals and p53 were also involved in apoptosis.

Materials and methods

Materials

DMAMI was synthesized according to our previous method.[12]

Treatment

To examine the effect of DMAMI-induced apoptosis in Colo205 cells, DMAMI was dissolved in dimethylsulfoxide (DMSO) and added to the culture media at final concentrations of 5, 10 or 20 μM for 2–6 h. The final concentration of DMSO in the medium was 0.1%.

Cell culture

Colo205 human colon cancer cell line derived from a colon cancer patient was obtained from ATCC (Rockville, MD). Colo205 cells were cultured in RPMI 1640 with 10% fetal bovine serum (Hyclone, Logan, UT) and 0.01 mg/ml gentamycin (Gibco, Grand Island, NY). Cells were kept in a CO_2 incubator with 5% CO_2 and 95% air at 37°C.

Preparation of cells for flow cytometric analysis

Cell cycle assay. The method of Landberg and Roos[13] was used. Drug-treated Colo205 cells were harvested and washed twice with phosphate-buffered saline (PBS), and the cell pellet was then mixed with 0.5 ml of lysing buffer containing 0.5% Triton X-100, 0.2 μg/ml EDTA and 1% bovine serum albumin in PBS. Cells were kept on ice for 15 min, followed by the addition of 3 ml methanol pre-cooled at −20°C and left at room temperature for 10 min. After fixation, cells were washed twice with PBS and the cell pellet incubated with 200 μl DNA staining medium [50 μg/ml propidium iodide, and 5 Kunitz/ml of RNase A (Sigma, St Louis, MO)] for 30 min at 4°C in the dark. Finally, cells were resuspended in 0.3 ml PBS and analyzed on a flow cytometer.

Annexin V binding assay. The cells were resuspended in 200 μl of 1 × binding buffer with 5 μl of enhanced Annexin V–FITC (final concentration: 0.5 μg/ml; Immunotech, Marseille, France) and 5 μl of propidium iodide (PI). The PI dye was used to gate for living cells and was added to each tube at a final concentration of 5 μg/ml. The cells were kept on ice for 10 min in the dark and then analyzed by flow cytometry.

Determination of free radicals by flow cytometry

In order to determine the DMAMI-induced changes in superoxide and peroxide contents in Colo205 cells, the method of Carter *et al*. was used.[14] Cells were trypsinized and washed twice with sterile HBSS, then resuspended in sterile-filtered HBSS solution with Ca^{2+} and Mg^{2+} (Gibco) containing 0.22% glucose, 2 mM glutamine and 1% bovine serum albumin. Cells were then mixed with either 20 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Eastman Kodak, Rochester, NY) or 10 μM hydroethidine (HE; Molecular Probes, Eugene, OR) and incubated in 37°C for 20 min. At the end of incubation, cell suspensions were kept in an ice bath (4°C). PI (10 μg/ml) was added to the tubes for gating the viable cells containing DCFH-DA and incubated for 10 min. Samples were analyzed by flow cytometry. Data were expressed as percentage of gated cells.

Measurement of $\Delta\Psi_m$ by flow cytometry

Alterations in the $\Delta\Psi_m$ were analyzed by flow cytometry using the $\Delta\Psi_m$-sensitive dye JC-1 (Molecular Probes, Eugene, OR).[15,16] Cells were harvested, washed once in PBS, resuspended in medium and

incubated with 1 μM JC-1 at 37°C for 10 min. Stained cells were then washed once in PBS and held at 4°C until evaluated by flow cytometry. Forward scatter (FSC) versus side scatter (SSC) was used to gate the viable population of cells. JC-1 monomers emit at 527 nm (FL-1 channel) and J-aggregates emit at 590 nm (FL-2 channel).

Flow cytometry

Cells (10 000) were analyzed on a Becton Dickinson (San Jose, CA) FACScan flow cytometer using an argon ion laser (15 mW) with the incident beam at 488 nm and red fluorescence (propidium iodide) was collected through a 585 nm filter. The data were analyzed using Lysys II software on a HP-310 computer.

Staining

Cells were fixed on gelatin-coated slides using Cytospin (Shandon, Astmoor, UK) and stained with hematoxylin & eosin, and then dehydrated through graded ethanol, cleared with xylene, mounted and examined.

Analysis of caspase-3, -8 and -9 expression by Western blotting

Cell lysates were prepared by the caspase-3 assay kit (PharMingen, San Diego, CA). Equal amounts of proteins (50 μg) were separated by 15% SDS–PAGE and then electrotransferred to nitrocellulose membranes which were subsequently blotted using anti-caspase-3 (Imgenex, San Diego, CA), -8 (R & D Systems, Minneapolis, MN) or -9 (Imgenex) antibodies and visualized by enhanced chemiluminescence.

Antitumor activity of DMAMI in the SCID mice tumor xenograft model

The SCID mice were obtained from the animal center of National Taiwan University Hospital (Taipei, Taiwan) and housed in a laminar flow room under sterile conditions with the temperature maintained at 25 ± 2°C and light controlled at 12 h light/12 h dark. Colo205 cells were harvested and resuspended in serum-free RPMI 1640 medium. Cells were adjusted to 1×10^7 cells/ml and 0.1 ml of cell suspension was inoculated per site s.c. in flank regions of 6- to 8-week-old male SCID mice. DMAMI was dissolved in olive oil (57.5 mg/ml). At day 10 after tumor cell inoculation, the tumor received an intratumoral injection of a single dose of 230 mg/kg DMAMI. The control group received an olive oil vehicle. Tumor size and body weight were monitored weekly. Tumor size was measured using a vernier caliper. Tumor areas were calculated using the formula: $(\text{length}/2 \times \text{width}/2) \times \pi$.[17] At the end of each experiment, mice were sacrificed by CO_2 gas, and tumors, livers, kidneys and lungs were collected, fixed, embedded and stained with hematoxylin & eosin. Six mice per group were used in each experiment. In another experiment, the DMAMI-induced morphological change in the tumor region was examined histologically at 2, 4 and 6 h after injecting the tumor with DMAMI (230 mg/kg, intratumoral, once).

Statistical significance between control and treated group was evaluated by using Student's *t*-test. $p < 0.05$ was considered as statistically significant.

Results

DMAMI induced morphological changes in Colo205 cells

Optical microscopy revealed that staining of DMAMI-treated Colo205 cells showed chromatin condensation, reduction in size and loss of membrane integrity at 6 h after DMAMI (10–20 μM) treatment (Figure 1).

DMAMI initiated cell cycle arrest and induced apoptosis in Colo205 cells

The effect of DMAMI on the cell cycle progression of Colo205 human colon cancer cells was examined at varying doses and time points by flow cytometry. Treatment of Colo205 cells with 20 μM DMAMI for 4–6 h resulted in a reduced population of G_2/M cells and the cells were accumulated in the G_0/G_1 phase (Figure 2A). With continued treatment, the typical apoptotic cells (sub-G_0/G_1 cells) with decreased DNA content were increased from 2.5 to 42.8% at 24 h (Figure 2B).

In early stages of apoptosis, the membrane phosphatidylserine (PS) is translocated from the inner to the outer plasma membrane, thereby exposing PS to the binding of Annexin V.[18]

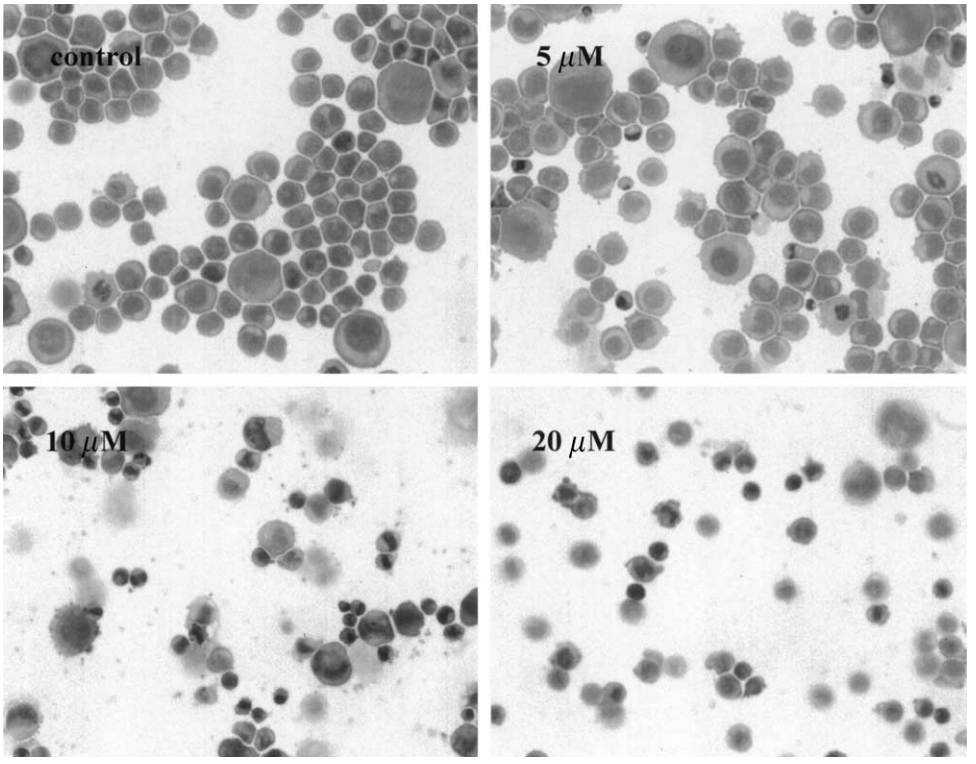

Figure 1. The effect of DMAMI on cell morphology of Colo205 cells. Cells were treated with 5,10 and 20 μM DMAMI for 6 h. Vehicle controls and DMAMI-treated cells were then 'cytospun' and stained with hematoxylin & eosin as described in Materials and methods.

Therefore, FITC-labeled Annexin V is often used as a probe for early apoptosis. For analysis of the early apoptosis induced by DMAMI, we examined the cells using Annexin V-conjugated FITC by flow cytometry. It was found that the vehicle-treated cells (control) were primarily Annexin V–FITC and PI^-, indicating that the cells were viable and were not undergoing apoptosis. The PI^- cells were gated for analysis of Annexin V binding. After treatment with 10–20 μM DMAMI for 2–6 h, the levels of Annexin V binding showed increases of 10.9–51.1% as compared to 1.9–2.8% of control (Figure 3). There were no significant changes in the level of Annexin V after Colo205 cells were treated with 5 μM DMAMI for 2–6 h (Figure 3). These results suggest that DMAMI-induced cell death appears to go through cell apoptosis.

The effect of DMAMI on the level of superoxide and peroxide in Colo205 cells

In some cases, apoptosis is accompanied by an elevation of the cellular levels of reactive oxygen intermediates (ROI), suggesting that ROI can act as mediators of apoptosis.[19] In order to examine whether the treatment of DMAMI affected the level of superoxide and peroxide in Colo205 cells, we examined the cells using HE and DCFH-DA as probes by flow cytometry. Figure 4(A) shows that the superoxide level was decreased in Colo205 cells after treatment with 10–20 μM DMAMI for 4 h. In comparison, the peroxide level was increased after treatment of Colo205 cells with 20 μM DMAMI at 2–6 h (Figure 4B).

Caspase activation in Colo205 cells

Caspases are activated during apoptosis by proteolytic processing at specific aspartate cleavage sites.[20] Caspase-3, -8 and -9 are commonly activated by numerous apoptotic stimuli. To assess whether the cytotoxic effect of DMAMI-treated Colo205 cells led to the activation of caspase-3, -8 and -9, cells were analyzed by immunoblotting with anti-caspase-3, -8 and -9 antibodies. The antibodies bind to a conformational epitope of these caspases which is exposed by activation-induced cleavage of pro-caspases. Activation of pro-caspase-3, -8 and -9 is

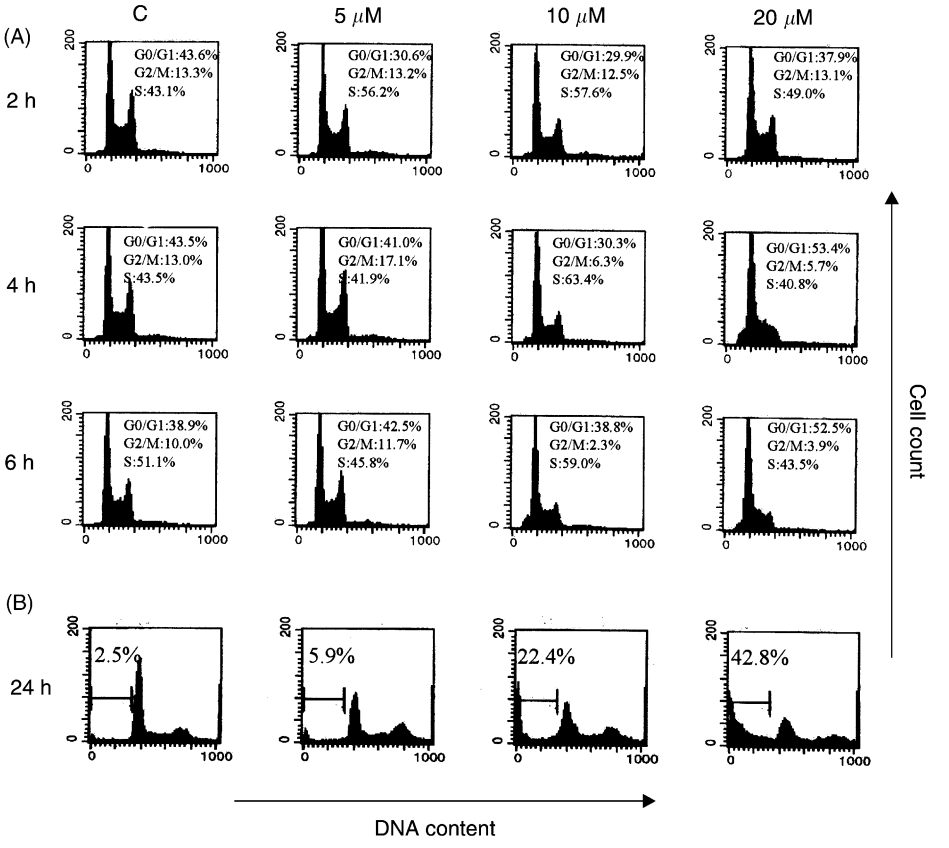

Figure 2. The effect of DMAMI on cell cycle progression in Colo205 cells. Cells were treated with 5, 10 and 20 μM DMAMI for (A) 2, 4 or 6 h and (B) 24 h prior to DNA histogram analysis by flow cytometry. C: vehicle control.

seen as a loss of their precursor or as the detection of their active form. The level of pro-caspase-3, -8 and -9 showed a decrease at 2 h after 10–20 μM DMAMI treatment. These decreases further increased at 4 and 6 h after DMAMI treatment of Colo205 cells (Figure 5). Thus, it appeared that the activation of caspases was associated with cell death.

DMAMI induced reduction of $\Delta\Psi_m$ in Colo205 cells

A decline of the $\Delta\Psi_m$ may be an early event in the process of cell death, on the basis of evidence demonstrating that escape from apoptosis is associated with increased $\Delta\Psi_m$, whereas activation of apoptosis cascades may be linked to disruption of the $\Delta\Psi_m$.[21–23] Therefore, we determined $\Delta\Psi_m$ by FACS analyses at various times after DMAMI treatment in Colo205 cells. For this purpose we used the $\Delta\Psi_m$-sensitive probe JC-1, which forms monomers (green fluorescence) at low membrane potential and J-aggregates (red fluorescence) at higher membrane potential. In comparison with that of untreated cells, monomeric JC-1 was found to increase from 0.5 (control) to 69.5% in 20 μM DMAMI-treated cells at 2 h. By 6 h, there was a dramatic increase (86.9%) of monomeric JC-1 in cells treated with 20 μM DMAMI as compared to control (0.8%) (Figure 6). A significant decrease in J-aggregates (from 95.8 to 78.2%) was evident as early as 2 h after exposure to 20 μM DMAMI. By 6 h, there was a dramatic drop of the red fluorescence (from 94.1 to 24.2%), indicating a loss of $\Delta\Psi_m$. These results suggest that initiation of growth arrest and apoptosis of Colo205 cells by DMAMI is associated with changes in $\Delta\Psi_m$.

Effects of DMAMI on the *in vivo* growth of Colo205 cells

To determine DMAMI antitumor efficacy *in vivo*, we implanted Colo205 cells s.c. in SCID mice and when the tumors were palpable (8–10 mm^2) the mice were either treated with vehicle control or DMAMI. Treatment of SCID mice with a single dose of DMAMI

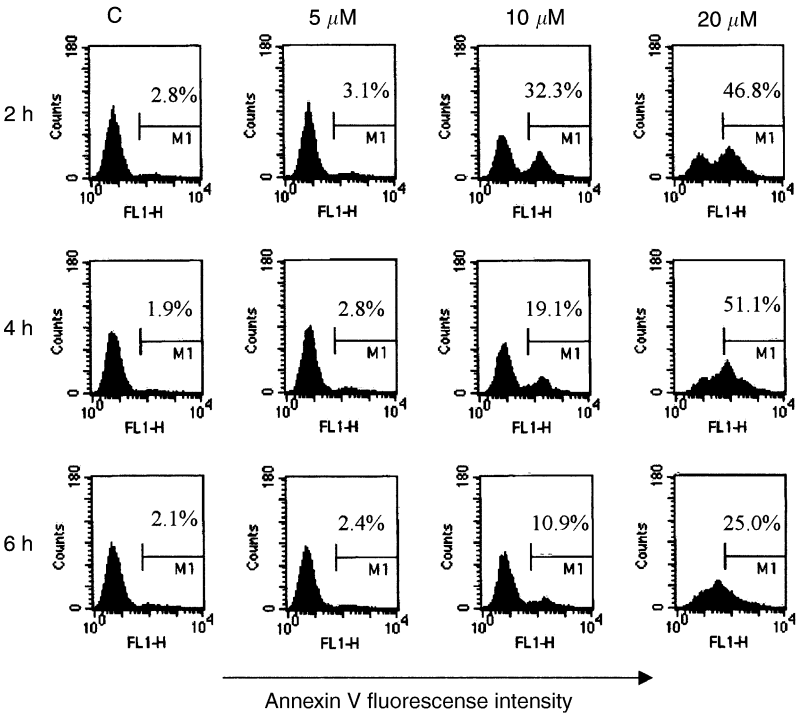

Figure 3. Flow cytometric analysis of Annexin V staining of Colo205 cells treated with 5, 10 and 20 μM DMAMI for 2, 4 or 6 h. DMSO was used as vehicle control. FL1-H represents Annexin V fluorescence intensity. The percentage represents the Annexin V–FITC$^+$ (PI$^-$) cells. Results are from a representative experiment of three separate experiments.

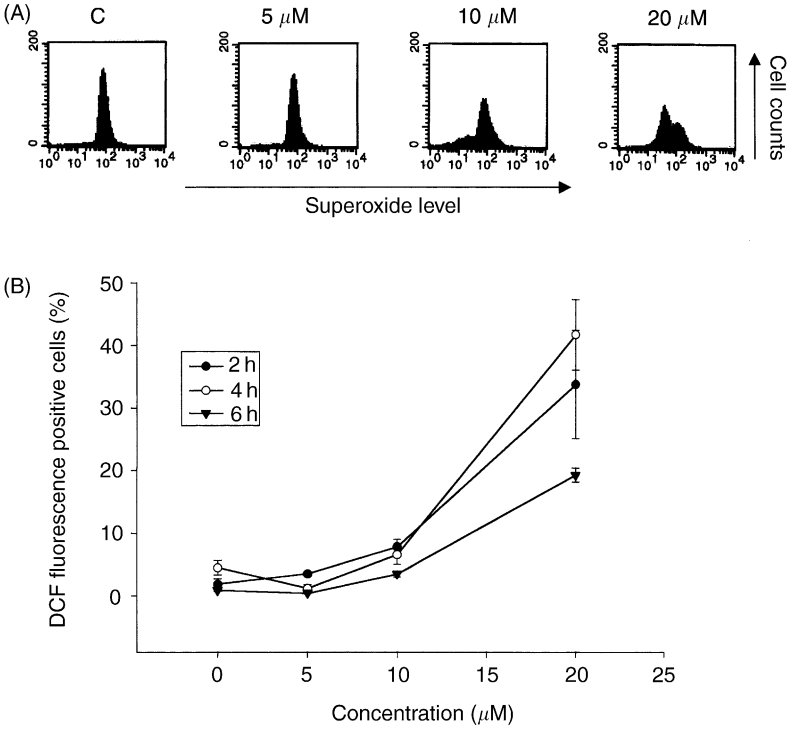

Figure 4. The effect of DMAMI on the formation of (A) superoxide and (B) peroxide radicals in Colo205 cells. Cells were treated with 5, 10 and 20 μM DMAMI for 2, 4 or 6 h. Vehicle controls and DMAMI-treated cells were then stained with hydroethidine (HE) or DCFH-DA and analyzed by flow cytometry as described in Materials and methods. Data are shown as the mean $\pm$ SEM from duplicate samples of three independent experiments.

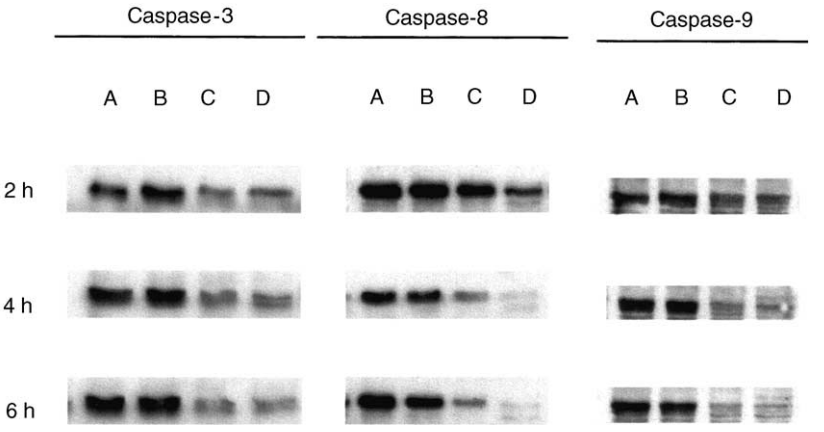

Figure 5. Activation of caspase-3, -8 and -9 in Colo205 cells. Cells were treated with (A) 0.1% DMSO, and (B) 5, (C) 10 and (D) 20 μM DMAMI for 2, 4 or 6 h and analyzed by Western blotting as described in Materials and methods. Activation of pro-caspase-3, -8 and -9 is seen as a loss of their pro-forms. Equal loading was determined by staining the membrane with GelCode Blue Stain reagent. Pictures are representative from one of at least three studies.

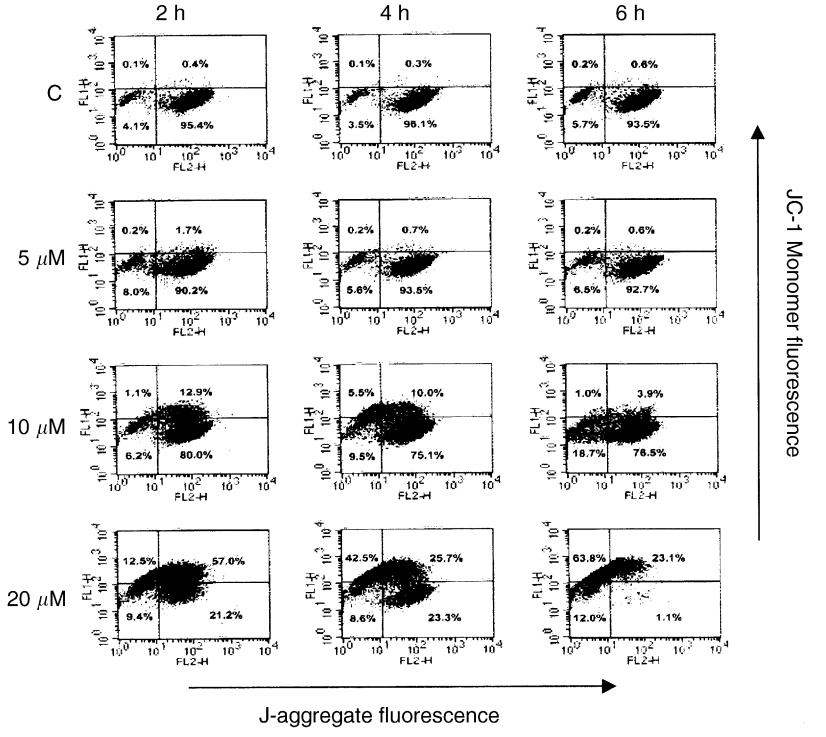

Figure 6. DMAMI-induced mitochondrial membrane potential ($\Delta\Psi_m$) reduction in Colo205 cells. Cells were treated with 5, 10 and 20 mm DMAMI for 2, 4, or 6 h. Vehicle control and DMAMI-treated cells were then stained with JC-1 and analyzed by flow cytometry as described in Materials and methods. Bivariate plots of green (FL1-H) versus red (FL2-H) fluorescence were used as an estimate of $\Delta\Psi_m$. Live cells were gated on the basis of their forward/side scatter characteristics. $n=10\,000$ cells/analysis. Three separate experiments were conducted that produced results similar to those shown here. As a control, $\Delta\Psi_m$ was measured in the presence of 0.1% DMSO.

(230 mg/kg) not only reduced the tumor incidence from 100% (six of six) to 33% (two of six), the tumor size was also significantly ($p<0.01$) reduced in mice as compared to control groups at the 4th week. Table 1 shows that tumors from control animals grew to an average size of $61.2\pm6.9\,mm^2$ which had

Table 1. The effect of single-dose treatment with DMAMI on the Colo205 human colon tumor xenograft

Group	*n*	Dose	Tumor size (mm^2) and incidence at weeks				
			0	1	2	3	4
Control	6	vehicle	8.5 ± 1.8[a] (6/6)	26.1 ± 3.9 (6/6)	39.6 ± 5.0 (6/6)	59.3 ± 6.3 (6/6)	61.2 ± 6.9 (6/6)
DMAMI	6	230 mg/kg	10.5 ± 1.2 (6/6)	–[b]	–[b]	22.5[c,e] (1/6)	19.3 ± 7.9[d,e] (2/6)

Cell suspensions of Colo205 were implanted s.c. in to the mice when the tumor size reached about 8–10 mm^2, DMAMI was injected once intratumorally.
[a]Average tumor size ± SE.
[b]At 1–2 weeks after single dose DMAMI treatment, hemorrhagic necrosis expanded and became a black spot.
[c]Recurrent tumor size.
[d]Average recurrent tumor size ± SE.
[e]$p < 0.01$.

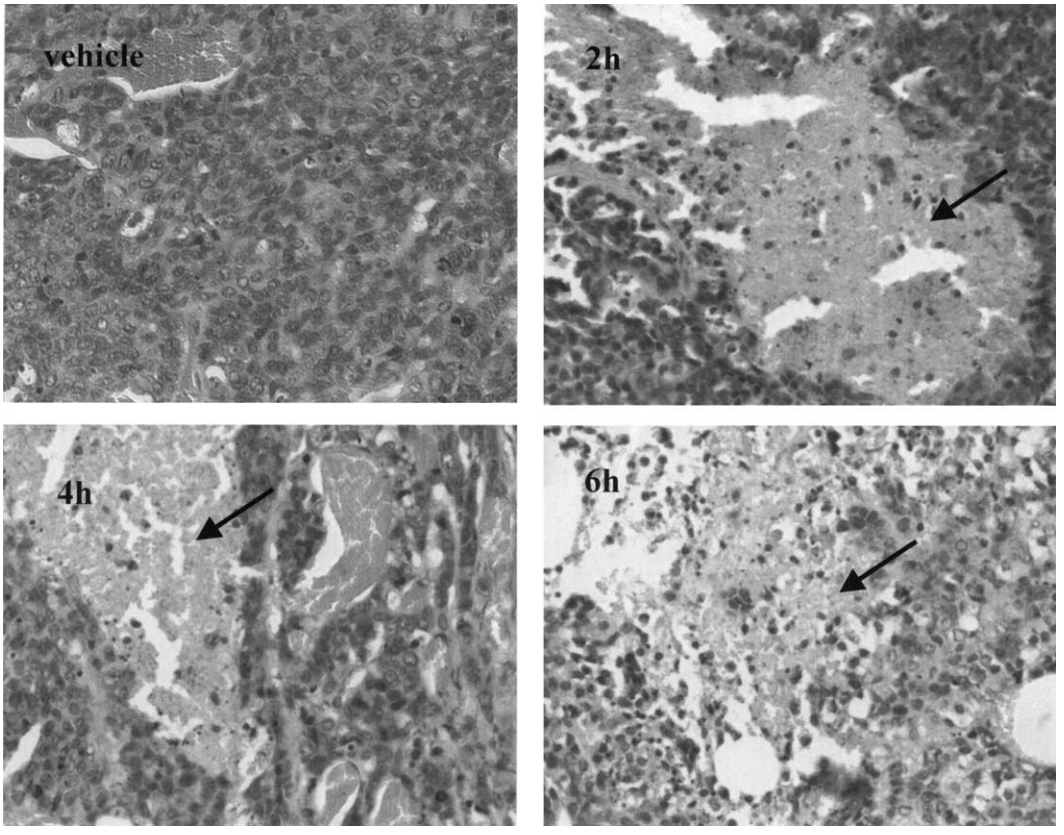

Figure 7. Morphological changes of tumor tissue in SCID mice at 2, 4 and 6 h after treatment with 230 mg/kg DMAMI.

increased about 7.5-fold at the 4th week. In contrast, tumors from DMAMI-treated animals grew to an average size of 19.27 ± 7.93 mm^2 ($n=2$) and four of six mice remained tumor free.

A s.c. single-dose challenge of DMAMI (230 mg/kg) in SCID mice produced no obviously acute toxicity. No significant reduction in body weight was found in DMAMI-treated mice. In addition, no tissue damage was observed in liver, lung and kidney after examination of the tissue slices stained with hematoxylin & eosin. We also compared the morphological changes of tumor tissue in SCID mice at 2, 4 and 6 h after treatment with 230 mg/kg DMAMI (intratumoral). Figure 7 shows that the tumor tissue in SCID mice revealed extensive destruction at 2–6 h after treatment with DMAMI. Figure 8 shows the DMAMI-treated tumors had scar formation as compared to the non-treated tumors.

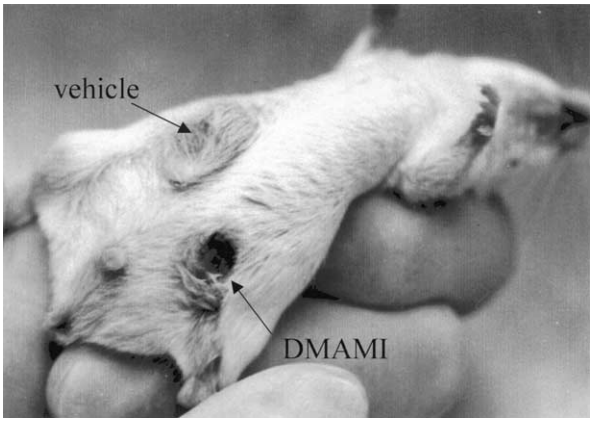

Figure 8. Hemorrhagic necrosis is evident at 1 week after treatment with a single dose of DMAMI (230 mg/kg).

Discussion

We have previously found that DMAMI exerts strong growth inhibitory activities *in vitro* against several cancer cells lines, including colon cancer cells.[7] However, the detailed mechanism of the DMAMI-induced growth inhibition of colon cancer cells is not clear. In this report, we have demonstrated that DMAMI induced cell cycle arrest and apoptosis in Colo205 human colon cancer cells. We have shown that within 4–6 h of exposure, DMAMI mediated accumulation of cells in the G_1/S region, but 24 h after DMAMI treatment, a significant number of cells have entered apoptosis, localizing in the subdiploid DNA peak (Figure 2). Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. Our result demonstrated that a significant number of cells were Annexin V–FITC$^+$ and PI$^-$, indicating that the cells were in an early stage of apoptosis and still viable after treatment with DMAMI (Figure 3). It is interesting that we have also observed an increase of peroxide levels (Figure 4B) in DMAMI-treated Colo205 cells. It has been reported that H_2O_2 induces an externalization of membrane PS and cell death[24,25] that correlates with the effects of DMAMI on Colo205 cells. These results suggested that ROI may act as mediators of DMAMI-induced apoptosis. One mechanism which is consistently implicated in apoptosis is the activation of a series of cytosolic cysteine protease, the caspases. Synthesized as inactive precursors, caspases must be proteolytically cleaved to become active enzymes.[20] The subsequent caspase activation and cleavage of substrates can amplify pro-apoptotic signals and finally leads to apoptosis. Western blotting confirmed that the levels of pro-caspase-3, -8 and -9 were activated when Colo205 cells were exposed to DMAMI at 4–6 h (Figure 5), and the fragmented subunits of caspase-3, -8 and -9 were detected (data not shown). These results suggested that the caspase-3, -8 and -9 were activated by DMAMI.

Recent studies have shown that alterations of mitochondrial functions such as permeability transition play a major role in the apoptotic process induced by numerous apoptotic stimuli.[26,27] It has been proposed that permeability transition would allow the passive release of cytochrome *c* and other caspase-activating factors from the mitochondrial intermembrane space into the cytosol.[28,29] JC-1 is a carbocyanine with a delocalized positive charge. The membrane potential of energized mitochondria (negative inside) promoted a directional uptake of JC-1 into the matrix, also with subsequent formation of J-aggregates, so J-aggregate fluorescence was sensitive to transient membrane potential changes. We have found that $\Delta\Psi_m$ was reduced after treatment with DMAMI on Colo205 cells (Figure 6), and the decrease of J-aggregate fluorescence correlated with the activation of caspase-3, -8 and -9. Caspase activation also has the capacity to induce dissipation of $\Delta\Psi_m$,[16] and caspase-3 may mediate lethal signaling in a manner specific to stimuli and cell types.[24,25] Tissue homeostasis is maintained through the balance between proliferation and apoptosis. The defective apoptotic pathways play a pivotal role in the risk of mammary tumorigenesis.[30] Effective chemotherapeutic agents, therefore, may prove to be those that promote both growth arrest and apoptosis. We suggest that the DMAMI-induced apoptotic cascade is characterized by disruption of $\Delta\Psi_m$ (Figure 6), activation of cytosolic caspases (Figure 5) and, finally, the generation of cells with subdiploid DNA content (Figure 2). These data suggest that the molecular and biochemical mechanisms responsible for early alteration in mitochondrial activities may play a fundamental role in initiating or coordinating DMAMI-mediated cell cycle arrest and apoptotic cascades.

DMAMI treatment led to increases in peroxide levels and activation of caspases, supporting peroxide- or caspase-mediated apoptosis in response to treatment, and increases the susceptibility of Colo205 cells to undergo apoptosis. Maleimide derivatives are alkylating agents for proteins containing suitable sulfhydryl groups.[31] Alkylation of sulfhydryl groups results in the inactivation of the proteins. A mechanism which cannot be ruled out is a process which consists of binding of the maleimide followed by a conformational change which leads to an

inactive protein.[32] Our results showed that there is more than one pathway of DMAMI-induced apoptosis. We suggest that DMAMI may react with the sulfhydryl groups of proteins in tumor cells and the alkylated protein will lose its function. The results suggest that functional loss of alkylated protein represents the major mediator of cytotoxicity by DMAMI on Colo205 cells.

In this study, we also demonstrate that treatment of Colo205 tumor-bearing SCID with DMAMI not only reduced the tumor incidence, but also resulted in significantly decreased tumor size (Table 1). The recurrent tumors in two of six treated mice may be the result of incomplete killing of tumor cells with a single treatment of DMAMI. Consequently, tumor cells grow back later. An important observation is that an intratumoral injection of DMAMI (230 mg/kg) in SCID mice produced no acute toxicity. The DMAMI-induced tumor damage was found rapidly at 2–6 h (Figure 7) and at 48 h after DMAMI treatment a black spot (scar formation) covered the whole tumor area, representing an area of hemorrhagic necrosis (data not shown). This result indicates that the *in vivo* antitumor activity of DMAMI occurred rapidly and was correlated with the *in vitro* cytotoxicity of DMAMI. Taken together, these results suggest that DMAMI treatment of Colo205 tumor is a direct and effective anticancer therapeutic approach. It is important to develop anticancer drugs that can attack the selective target and accumulate in the desired tissue without systemic effects. Therefore, DMAMI is suitable for development as an anticancer drug for local administration without systemic effects.

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